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Ligand occupancy mimicked by single residue substitutions in a receptor: Transmembrane signaling induced by mutation

(signal transduction/bacterial chemotaxis/chemoreceptor/membrane protein/random oligonucleotide-directed mutagenesis)

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ABSTRACT **We used mixed, mutagenic oligonucleotides to create single amino acid substitutions in the bacterial chemoreceptor Trg. Mutagenesis was directed at a 20-residue segment of the periplasmic domain implicated in ligand recognition. Transmembrane signaling by the mutant receptors was assayed *in vivo* by monitoring adaptational covalent modification. Among 20 functionally altered but stable receptors there were two distinct signaling phenotypes. Insensitive receptors did not signal upon stimulation and thus appeared defective in productive ligand interaction. Mimicked-occupancy receptors exhibited transmembrane signaling without ligand. Many mimicked-occupancy receptors produced additional signaling upon ligand binding and in appropriate conditions mediated effective chemotaxis; most insensitive receptors did not. Like normal receptors with one binding site occupied, mimicked-occupancy proteins adapted to persistent transmembrane signaling by increased methylation and thus could respond to other stimuli. Signaling phenotypes were strikingly segregated by residue position. Substitutions mimicking ligand occupancy occurred in half the segment, and those creating insensitive phenotypes occurred in the other half. These observations could be related to the three-dimensional structure of the periplasmic domain of the Tar_s chemoreceptor. Insensitive substitutions occurred near the distal end of helix 1, where bulky protein ligands could interact; occupancy-mimicking substitutions were on the same helix at positions buried in the subunit interface between helices 1 and 1′. Thus perturbation of the interface induced transmembrane signaling, implicating changes at that interface in signal transduction, a conclusion consistent with differences in crystal structures of unoccupied and ligand-occupied Tar_s.**

Transmembrane receptor proteins transduce ligand recognition into intracellular signals. Binding of ligand to an extracytoplasmic domain alters the activity of a domain on the other side of the membrane. This process, called transmembrane signaling, is not understood for any receptor but is widely supposed to resemble allosteric phenomena in that binding at one site induces a conformational change that affects a distant site. Is it possible to separate generation of the conformational signal from ligand binding? In this report we describe single amino acid substitutions in the ligand-recognition domain of the bacterial chemoreceptor Trg that generate signaling in the absence of ligand.

Four homologous transmembrane receptors in *Escherichia coli* mediate chemotaxis to amino acids, sugars, and dipeptides. Information about receptors and the chemotactic system, as well as primary references for the following summary, can be found in recent reviews (1, 2). Trg, Tar, and Tap recognize, respectively, the ligand-occupied forms of galactose- and ribose-binding proteins, maltose-binding protein, and dipeptide-binding protein. Through this recognition of polypeptide ligands, the three receptors mediate taxis toward the corresponding small-molecule attractants. In addition, two receptors bind small molecules directly: Tar binds aspartate; Tsr binds serine. The receptors are homodimers of 60-kDa subunits. Each subunit has three domains, a periplasmic domain that contains the ligand-recognition site, a cytoplasmic domain that initiates intracellular signaling and mediates sensory adaptation, and a transmembrane region of two short segments that connect the two other domains. Since these receptors transduce ligand recognition into an intracellular signal, they are often called transducers. The signaling domain of a transducer influences flagellar rotation and thus motile behavior by controlling a protein kinase. This signaling domain contains four or five glutamic residues that are methylated by a specific methyltransferase and demethylated by a specific methylesterase. Appropriate changes in methylation levels of stimulated transducers mediate sensory adaptation.

In the aligned sequences of the four receptors, the cytoplasmic domains exhibit many amino acid identities and the periplasmic domains exhibit few. Yet mutational substitutions that affect ligand interaction cluster in two limited regions of the aligned sequences of the periplasmic domains (Fig. 1 *A* and *B*), implying a common organization of the ligand-binding region. We chose the first of those regions, corresponding to Trg residues 69–88, for detailed mutational analysis.

MATERIALS AND METHODS

Strains and Plasmids. CP362 and CP593 are derivatives of *E. coli* K-12 strain OW1 (10). CP362 is Δ(*tsr-7028*), Δ(*tar-tap*)*5201*, Δ(*trg-100*) and thus has no active transducer genes (11). CP593 is Δ(*trg-100*), Lac^+ and was derived from CP177 (4) by P1 transduction from a Lac^+ strain. pMG2 is a pUC13 derivative containing *trg* under control of its native promoter (11).

Mutagenesis and Strain Construction. A mixture of mutagenic 60-mers corresponding to *trg* codons 69–88 were synthesized by adding a total of 1.6% (1/60) of the other three nucleotide reagents to each reagent used for oligonucleotide synthesis (12, 13). The gel-purified oligonucleotides were used as primers for *in vitro* DNA synthesis directed by an M13mp11–*trg* hybrid produced from the *dut, ung* strain CJ236 (14, 15), and a sample of the reaction mixture was used to transform the Dut^+, Ung^+ strain JM101 (16). Transformants were screened by nucleotide sequencing of isolated M13 DNA (30% of randomly chosen plasmids contained one or more nucleotide substitutions) or by transferring the 2.2-kilobase *Hin*dIII–*Eco*RI fragment containing *trg* from the M13 construct to pUC13 (thus creating plasmids related to pMG2), transforming CP362, and testing transformants for ability to form chemotactic rings in response to galactose and

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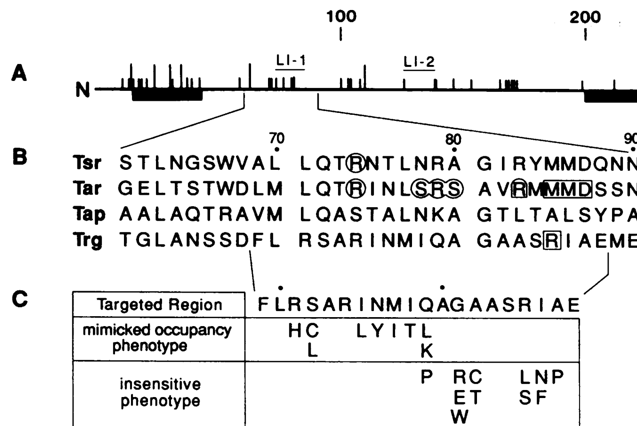

FIG. 1. Targeted mutagenesis of the ligand-recognition domain of Trg. (*A*) Aligned sequences of the four transducers of *E. coli* (3) from amino termini through second transmembrane segments. Tall and short vertical lines indicate positions of amino acid identity for all four or for three of four transducers, respectively. Black boxes indicate transmembrane segments, and LI-1 and LI-2 label regions implicated in ligand interaction. Adapted from ref. 2. (*B*) Aligned transducer sequences including ligand-interaction region 1. Position numbering is for Trg; corresponding residues in other sequences have numbers 10 lower because Trg has 10 extra amino-terminal residues. Residues implicated in ligand interaction by mutational analysis are circled for small-molecule interaction (serine or aspartate), boxed for binding-protein interaction or surrounded by a hybrid of the two shapes for both types of interaction. Data from refs. 4–9. (*C*) Mutational substitutions characterized in this work are grouped into the two phenotypic classes discussed in the text.

ribose. Strains with single copies of mutated *trg* genes integrated into the *lac* region were created by selecting for integration of pUC13–*trg* hybrids into *lac* in a temperature-sensitive *polA* strain, transducing the integrated plasmid to CP593, and screening for resolution and loss of the plasmid (4).

Immunoblots. Derivatives of CP362 harboring pUC13–*trg* hybrid plasmids were grown in tryptone broth at 35°C in the absence or presence of 15 mM ribose. At approximately 2.5×10^8 cells per ml (in the midst of logarithmic growth), a sample of 2.5×10^7 cells was added to trichloroacetic acid (10% final concentration). Precipitated material was subjected to polyacrylamide (11% total monomer, 0.073% *N,N'*-methylenebisacrylamide) gel electrophoresis (11), electroblotted onto nitrocellulose, treated with anti-Trg serum and a peroxidase-coupled secondary antibody (Bio-Rad), and incubated with hydrogen peroxide and 4-chloro-1-naphthol.

Other Procedures and Materials. Growth media (17) and semisolid agar swarm plates (18) were prepared as described. Standard procedures for manipulation of DNA were as described (19, 20). Nucleotide sequences were determined (21) using Sequenase (United States Biochemical).

RESULTS

Mutational Substitutions in a Ligand-Recognition Region. We used a mixture of mutagenic oligonucleotides to generate random nucleotide substitutions in the *trg* segment that codes for residues 69–88, a region implicated in ligand recognition (Fig. 1). Cells lacking chromosomal genes for the four transducers, but otherwise wild-type for chemotaxis, were transformed with pUC13 derivatives containing mutagenized *trg* genes. Individual transformants were tested for ability to form chemotactic rings in response to galactose and to ribose, appropriate segments of plasmids were sequenced to identify nucleotide substitutions, and cells were analyzed for content of transducer by immunoblotting with anti-Trg. This survey yielded a collection of 20 different mutated *trg* genes producing proteins with single amino acid substitutions (Fig. 1*C*) that created functional alterations without significant effects on protein stability.

Covalent Modification as an Assay for Intramolecular Signaling. We used measurements of methylation level to assay transmembrane signaling by altered Trg receptors. The essence of signaling is that a change in occupancy of the ligand-binding site in the periplasmic domain alters the state of the cytoplasmic domain. Two changes occur; one is transient, the other persistent. The transient change affects interaction of transducer with kinase; it is transient because an appropriate increase in methylation of the cytoplasmic domain causes a compensatory change in that domain which restores receptor–kinase interaction to the null state. The persistent change is the increased level of methylation. Thus transmembrane signaling *in vivo* from altered periplasmic domains to normal cytoplasmic domains can be examined by comparing extents of receptor methylation in unstimulated and stimulated cells, which we did using immunoblots. Previous work has shown that transducers are displayed by SDS/PAGE as an array of bands corresponding to different numbers of methyl groups per polypeptide chain (22–25). Transducer polypeptides with glutamates at all methyl-accepting sites migrate most slowly and, with the exception of one methyl-accepting position in Trg, each added methyl group results in a characteristic increase in electrophoretic mobility (11, 26) through an effect that is not entirely understood but may involve increased binding of SDS molecules. In any case, the population of transducers in unstimulated cells exhibits a characteristic electrophoretic pattern of slower migrating bands that reflects low numbers of methyl groups per polypeptide (on average, approximately one), while transducers in cells exposed to a saturating stimulus and allowed to adapt exhibit a pattern shifted to more rapidly migrating species corresponding to greater numbers of methyl groups (an average between two and three) (Fig. 2, WT lanes). The shift to predominantly faster migrating and thus more-methylated polypeptide species in cells adapted to continual, maximal occupancy of the transducer is diagnostic for functional signaling from periplasmic to cytoplasmic domain. The use of immunoblotting, rather than fluorography of [^{3}H]-methyl-labeled material, allowed signaling to be assessed without removing cells from growth conditions or inhibiting protein synthesis. This technique gave clear results for cells lacking chromosomal transducer genes and harboring a *trg*-containing plasmid. The 20 altered Trg proteins were examined for transmembrane signaling using this assay (Fig. 2).

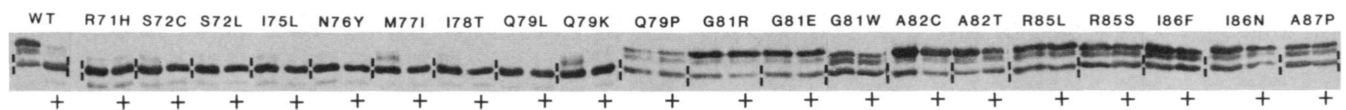

FIG. 2. Signaling by wild-type and altered Trg proteins. Methylation state was used to assay signaling *in vivo* by Trg proteins. The figure is a composite of immunoblots of strains producing the indicated forms of Trg as the only transducer in the cell. Only the region including transducer bands is shown: the lowest band in the leftmost lane has an apparent $M_r \approx 60,000$. For strains containing wild-type Trg (WT) or an altered Trg (indicated by the wild-type residue, residue number, and substituted amino acid) two conditions are shown: no Trg-mediated attractant (no symbol) and saturating levels of ribose (+). Patterns characteristic of the two phenotypic classes were also seen when representative proteins were examined using [^{3}H]methyl-labeled Trg and fluorography.

Two Distinct Signaling Phenotypes. All 20 mutant proteins differed from the wild-type transducer in the signaling assay. The 20 fell into two distinct classes. Within each class there was some variation, but almost no overlap with features of the other class. One class, designated insensitive, exhibited an unstimulated pattern that differed little from that of wild-type Trg, but unlike the normal transducer, showed little or no increase in methylation in the continued presence of a stimulating attractant. Thus it appears that signaling is defective in these altered transducers. This might reflect a defect in ligand binding or in generation of signaling by binding. The second class, designated mimicked occupancy, had a level of methylation in the absence of stimulation that was similar to the increased methylation of a normal transducer after maximal stimulation by a single ligand and subsequent adaptation. Thus the specific, single amino acid substitutions in the periplasmic domains of these mutant transducers induced an alteration in the cytoplasmic domain that mimicked the change usually caused by ligand occupancy. Six of the nine mimicked-occupancy receptors exhibited a distinct additional increase of methylation in response to ribose, indicating that these proteins were capable of transmembrane signaling in response to conventional ligand occupancy. As discussed in the next section, a more sensitive assay of receptor function revealed that all nine mimicked-occupancy receptors had at least some ability to mediate chemotaxis and thus also the capability of transmembrane signaling. In Fig. 2, pairs of patterns for the 20 altered transducers in the absence and presence of the attractant ribose are arranged by residue number. There is a striking segregation of signaling phenotypes. Substitutions at positions after residue 79 all exhibited the insensitive phenotype; those prior to 79 all exhibited the mimicked-occupancy phenotype. Depending upon the substitution, alterations at position 79 exhibited one or the other phenotype. Trg-R85H, previously characterized as defective in ligand interaction (4), exhibited an insensitive phenotype in the signaling assay, a result consistent with its position in the segregated distribution of phenotypes.

Mediation of Chemotaxis by Altered Transducers. The 20 altered Trg proteins were defective in comparison to the normal transducer in mediating chemotactic ring formation by cells harboring a *trg* gene on a multicopy plasmid but lacking other transducers. Since the chemotactic system is sensitive to relative amounts of component proteins, the ability of altered transducers to mediate taxis was examined in cells containing a single chromosomal copy of each transducer gene, including the altered *trg*. All these strains exhibited a swimming pattern of runs and tumbles. Tests of ability to form chemotactic rings revealed that the two classes of altered transducers identified by the signaling assay had rather different functional abilities (Fig. 3). Most of the insensitive class, including the previously characterized Trg-R85H (data not shown), were essentially unable to mediate formation of chemotactic rings, a defect consistent with the postulated disruption of ligand interaction. In contrast, mimicked-occupancy proteins mediated chemotactic ring formation with efficiencies from almost normal to distinctly but not completely defective. Thus several of the occupancy-mimicking substitutions had only minor effects on transducer function in an otherwise normal cell. This pattern was confirmed by quantitative capillary assays of chemotaxis (unpublished work).

DISCUSSION

By using covalent modification as an assay for transmembrane signaling, we identified two distinct signaling phenotypes for Trg receptor proteins containing single amino acid substitutions in a region implicated in ligand interaction. Fig. 4 summarizes our interpretation of these phenotypes.

Mimicking Ligand Occupancy. Mutational substitutions that induce or favor the signaling conformation of the periplasmic domain of a chemoreceptor would mimic ligand occupancy. Using our signaling assay we have identified such substitutions in Trg. A distinctive feature of occupancy-mimicking substitutions is that receptor function might be relatively unperturbed. The logic is as follows. A normal receptor with two separate ligand-binding sites—for example, Tar—mediates effective taxis to one ligand even though it is saturated with the other (27). This is because maximal occupancy at one site is adapted to by an appropriate increase in methylation, resetting the cytoplasmic domain to a null signaling state poised for response to occupancy of the other site. Analogously, mimicked-occupancy Trg proteins adapt to mutationally induced transmembrane signaling by increased methylation and would thus be poised for response to occupancy at the ligand-binding site. In fact, some mimicked-occupancy receptors were almost as effective as normal Trg in mediating chemotaxis when present in balanced amounts in otherwise wild-type cells. Other mimicked-occupancy receptors exhibited more or less severe functional defects. The defects might occur because the substitution not only created signaling but also disturbed a feature important for receptor function, or because the substitution created an extreme of the signaling conformation, bringing signaling capacity close to saturation. Mimicked-occupancy mutants resemble some biased signal mutants described in Tsr (28), but are distinctly different from locked signal mutants characterized extensively in Tsr (29) and also identified in Tar (30)

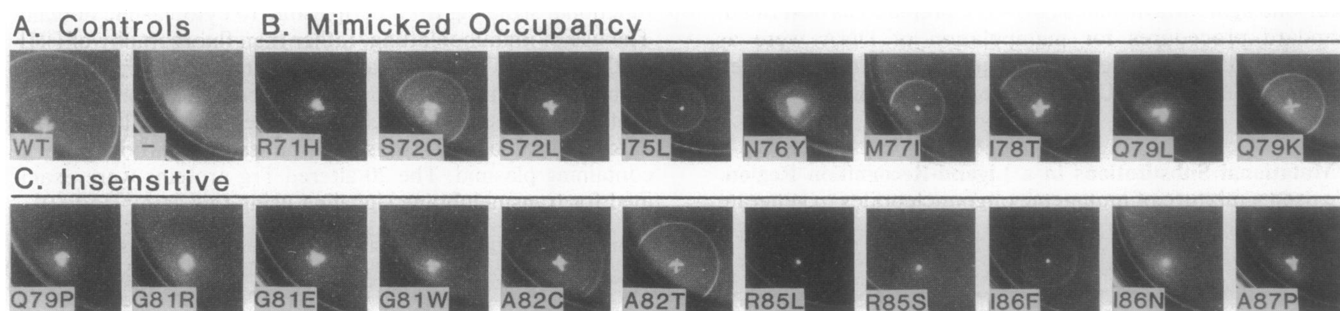

FIG. 3. Chemotaxis mediated by wild-type and altered Trg proteins. Motile, growing cells carrying a single chromosomal copy of *trg* in the *lac* region and normal chromosomal genes for all other chemotaxis components were inoculated into semisolid agar plates containing minimal salts, required amino acids, and a limiting amount (0.1 mM) of ribose and were incubated at 35°C for 17 hr. (*A*) The strain containing wild-type *trg* (WT) formed a sharp, dense ring of cells that moved out from the point of inoculation in response to the gradient of ribose formed by metabolism of the sugar. A strain deleted of trg (−) formed a diffuse, slow-moving disk as the result of random movement of highly motile but nonresponding cells. (*B* and *C*) Strains containing altered Trg proteins (notations as for Fig. 2) are displayed in the order of substitution position and labeled according to phenotypic class. The differences between strains in ring size and sharpness were reproducible in repeated analyses.

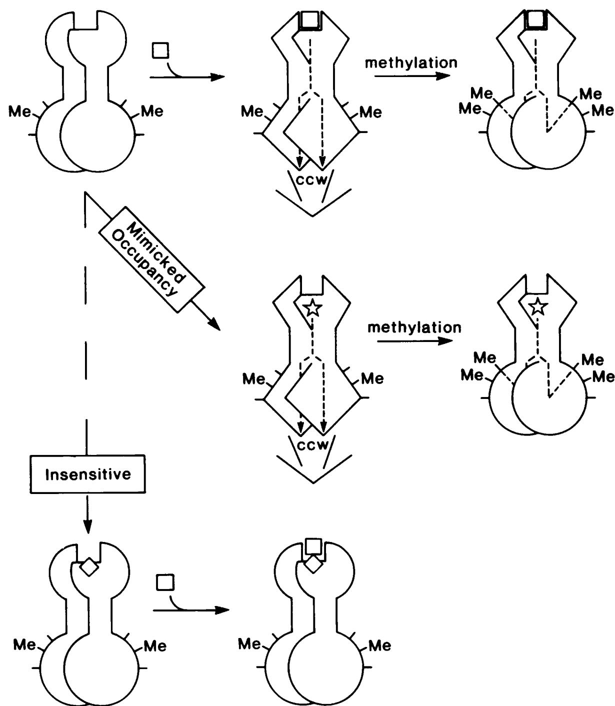

FIG. 4. Cartoon representation of signaling phenotypes generated by single amino acid substitutions in the ligand-interaction region of Trg. The top row diagrams for a wild-type receptor the processes of excitation upon ligand binding, and adaptation mediated by increased methylation. The middle row shows the effect of an occupancy-mimicking substitution and the compensation of mutationally induced transmembrane signaling by increased methylation. The bottom row illustrates the effect of an insensitive substitution, blocking productive ligand interaction and thus excitation. ccw, Counterclockwise (referring to flagellar rotation).

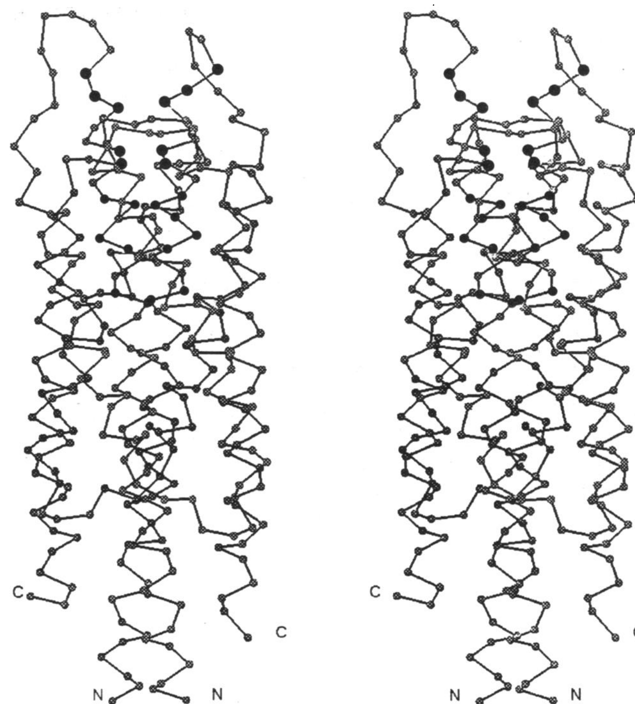

FIG. 5. Stereoview of the C^{α} atoms of the ligand-occupied form of the periplasmic domain of Tar_S. Positions marked correspond to mutational changes in Trg that confer insensitive (large dots) or mimicked-occupancy (small dots) phenotypes. This image was created by using Insight 2 by Biosym Industries.

and Trg (31). Unlike mimicked-occupancy receptors in which increased methylation compensates for mutationally induced signaling, receptors altered by locked signal mutations exhibit constant signaling by the cytoplasmic domain in spite of altered methylation that would normally balance ligand occupancy. This phenotype is conferred by substitutions at many positions in the cytoplasmic domain but by none in the periplasmic domain. Thus unlike occupancy-mimicking mutations, these mutations do not induce transmembrane signaling.

Substitutions that confer the mimicked-occupancy phenotype have the striking property of uncoupling transmembrane signaling from ligand recognition. Induction of signaling by single substitutions in the periplasmic domain suggests that the signaling conformation of the domain is relatively accessible energetically from the nonsignaling conformation. Several conservative substitutions that have no general disruptive effect on structure can shift the balance between nonsignaling and signaling conformations to favor the latter. It may be that the methylation state of unstimulated transducers (approximately one modification per protein) reflects the probability of attaining the signaling conformation in the absence of ligand.

Ligand Interaction and Intramolecular Signaling. The positions of insensitive mutations identify residues important for productive interaction with the protein ligands of Trg. These positions correspond closely to those implicated in interaction of Tar with maltose-binding protein (Fig. 1). The basis for the insensitive phenotype might be disruption of ligand binding, uncoupling of binding from induction of signaling, or creation of a negatively signaling conformation that would cancel ligand-induced signaling. Previous characterization of Trg-R85H (4) indicated that the defect in this insensitive receptor was induction of signaling by binding rather than in affinity of receptor for ligand, and thus had the character of the latter two possibilities. Ligand occupancy is mimicked by substitutions adjacent to the region involved in ligand interaction. The location suggests the following explanation for mimicry. Residues at which substitutions mimic ligand occupancy are intimately involved in structural interactions altered by the conformational change created upon ligand binding. This change is usually induced or stabilized by bound ligand; in mimicked-occupancy transducers it is favored by alternative interactions of the substituted side chain. The recently determined three-dimensional structure of the periplasmic domain of the aspartate receptor from *Salmonella typhimurium*, Tar_S (32), provides a structural context in which to explore these notions. A stereo diagram of the C^{α} backbone of this structure is shown in Fig. 5.

Signaling Phenotypes and Transducer Structure. The periplasmic domain of Tar_S is a dimer of two four-helix bundles arranged as parallel subunits with contacts at each end between helices 1 and 1′. The structure approximates a cylinder 40 Å in diameter and >70 Å long. Aspartate binds in the membrane-distal subunit interface, with interactions to Arg-64 of helix 1, Arg-69′ and Arg-73′ of helix 1′ (these are the arginines circled in the Tar sequence in Fig. 1B) and several residues of helix 4. Tar_S is closely related to Tar from *E. coli*, and thus alignment of the transducer sequences from *E. coli* (3) could be used to align sequences of the periplasmic domains of Tar_S and Trg with only two single-residue gaps, one each in helices 2 and 3.

Examination of positions in the Tar_S structure that correspond to the mutational substitutions in Trg (Fig. 5) suggests a structural basis for the two distinct phenotypes and their segregated distribution. The substitutions that mimic ligand occupancy, indicated by small dots in Fig. 5, are in the segment of helix 1 at which 1–1′ contacts form the membrane-distal interface between the subunits of the dimer. The side

chains at these positions are buried: those at positions 71, 76, 77, and 78 exhibit no solvent-accessible surface, residue 75 has 1% of its area accessible, and residues 72 and 79 have 10% accessible (S.-H. Kim, personal communication). The positions of substitutions conferring insensitive phenotypes (large dots in Fig. 5) are in the most membrane-distal segment of helix 1, as it extends beyond the four-helix bundle, and in the first two residues of the loop that connects helices 1 and 2. Solvent accessibilities vary from low values of 10, 0, and 0% for the residues within the helix, positions 79, 81, and 82, to substantial values of 20, 36, and 56% for the final residue in the helix, position 85, and the two in the loop, residues 86 and 87 (S.-H. Kim, personal communication). Thus, residues that can be changed to create an insensitive phenotype are appropriately placed to perform the suggested role in productive ligand interaction. Solvent-exposed residues could participate in interaction with bulky, binding-protein ligands. Buried residues that are sites of insensitive substitutions are structurally coupled to the solvent-exposed residues by one or two helical turns and thus could be involved in coupling ligand interaction to the conformational change, a coupling that could be disrupted by substitution with a larger side chain unable to fit in the available space (G81R, G81E, G81W, A82C, and A82T) or with a helix breaker (Q79P). The side chains at positions where substitutions mimic ligand occupancy are not available for interaction with bulky ligands and thus are not appropriately placed to be in the binding site *per se*. This makes it unlikely that mimicked occupancy is generated because such a substituted side chain interacts with other Trg residues as if it were a side chain of the polypeptide ligand. Instead the signal must be created because of altered interactions of buried residues. This is consistent with the mimic of ligand binding occurring at the level of the conformational change that initiates transmembrane signaling. What is the nature of that conformational change? The periplasmic domains of unoccupied and aspartate-occupied Tar_s differ in only the relative positions of subunits, suggesting initiation of intramolecular signaling by a scissors movement of helices 1 and 1′ (31). Substitutions that mimic ligand occupancy could alter side chain packing in the interface to favor or stabilize the subunit disposition usually induced by ligand interaction. The striking segregation of phenotypes along the primary structure of the targeted region would thus reflect segregation of functional interactions along an extended helical structure.

Occupancy-mimicking substitutions identify the membrane-distal, subunit interface between helices 1 and 1′ as an important structure in intramolecular signaling and demonstrate that modest changes in residues buried in that interface are sufficient to generate a transmembrane signal. This identification is based on *in vivo* assays of activities of intact receptors and alignment of the Trg sequence with the three-dimensional structure of the periplasmic domain of Tar_s. An important role for this interface in signaling was suggested by the conformational difference between the three-dimensional structures of unoccupied and ligand-occupied Tar_s (32). However, the authors noted that the relatively subtle difference between the two forms could be an artifact of different crystal packings or an incomplete reflection of more extensive differences damped by investigator-imposed crosslinking of the two subunits. These concerns do not apply to our results, and thus the combination of genetic and crystallographic studies provides a strong argument that changes in the dimer interface are involved in the primary event of intramolecular signaling. How does a change in the 1–1′ helix interface cause a corresponding transmembrane signal? One possibility is coupled movement of the transmembrane extensions of those helices (32). However, an *in vitro* study of Tar_s indicates that at least some aspects of transmembrane signaling can occur in an incomplete dimer with one subunit intact but the other only a periplasmic domain (33). Thus intramolecular signaling initiated in the 1–1′ helix interface can be conveyed across the membrane by a receptor with a transmembrane extension of only one of the 1–1′ pair, perhaps by relative changes in the two transmembrane segments of the single intact subunit. Characterization of other substitutions that mimic the effect of ligand occupancy may provide insight into this issue.

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